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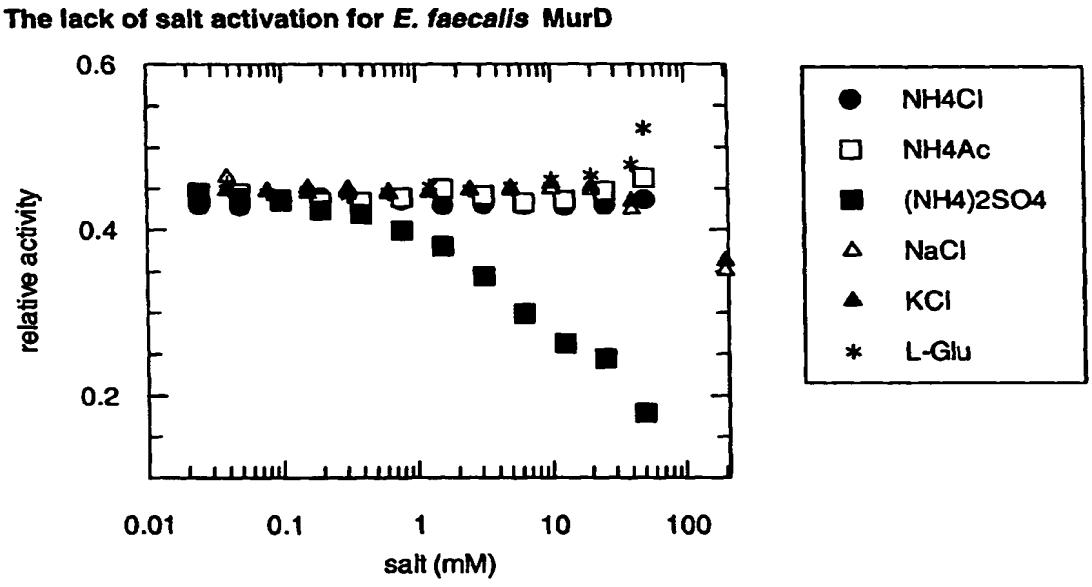
(54) Title: SCREENING ASSAY TO IDENTIFY INHIBITORS OF THE MURD ENZYME USING AN ACTIVATOR-INDEPENDENT MURD ENZYME



(57) Abstract: The use of an activator-independent MurD enzyme in a screening assay to identify inhibitors of the enzyme, which assay comprises contacting the enzyme with a test compound in the presence of enzyme substrates and appropriate buffers and detecting any modulation of enzyme activity by the test compound.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

SCREENING ASSAY TO IDENTIFY INHIBITORS OF THE MURD ENZYME USING AN ACTIVATOR-INDEPENDENT MURD ENZYME

The present invention relates to improved screening assays and in particular to the use of activator-independent forms of the murein biosynthesis enzyme MurD, such as from *Enterococcus faecalis (E. faecalis)*. Such screening assays are used to identify and characterize modulators of the MurD enzyme.

Interest has been shown in the murein (Mur) biosynthesis pathway in bacteria, this is a key component of bacterial cell wall synthesis. Enzymes in this pathway are potential targets for broad-spectrum and selective antibacterial agents.

The bacterial enzyme MurD (UDP-N-acetylmuramyl-L-alanine:D-glutamate ligase catalyses the attachment of D-glutamate to a cytoplasmic peptidoglycan precursor, UDP-N-acetylmuramyl-L-alanine. This reaction results in the formation of a peptide linkage between the amino function of D-glutamate and the carboxyl terminus of UDP-N-acetylmuramyl-L-alanine. A stoichiometric comsumption of ATP supplies the energy needed for this peptide bond formation resulting in generation of ADP and orthophosphate.

Walsh et al (Journal of Bacteriology, Sept 1999, 181, No.17, 5395-5401) have examined the biochemical properties of the Mur D enzyme from two gram-negative bacteria, i.e. *Escherica coli*, and *Haemophilus influenzae*, and two gram-positive bacteria i.e. *Enterococcus faecalis* and *Staphylococcus aureus*. They established data regarding the biochemical properties of these enzymes and discussed similarities and differences between them, in particular with regard to salt-activation of the gram-negative bacteria. They report that the differences observed between the gram-positive and the gram-negative bacteria indicate that the two gram-negative bacteria may apply a more stringent regulation of cell wall biosynthesis at the early stage of the peptidogylcan biosynthesis pathway than do the two gram-positive bacteria. However, the skilled reader is aware that the substrate purification procedure used by Walsh et al cannot remove all salts that may function as enzyme activators. Therefore it is not possible to draw meaningful conclusions as to the salt-dependenc or lack of salt dependence of the two gram-positive bacteria.

Indeed, we have now found that the MurD enzyme from the gram-positive bacterium *Staphylococcus aureus* is also salt-activated. This led to our discovery that the *E. faecalis* MurD enzyme has unique properties which make it possible to devise improved screening assays using an activator-independent MurD enzyme.

Therefore in a first aspect of the present invention, we provide the use of an activator-independent MurD enzyme in a screening assay to identify inhibitors of the enzyme, which assay comprises contacting the enzyme with a test compound in the presence of an enzyme substrate and appropriate buffers and detecting any modulation of enzyme activity by the test compound.

By "activator-independent" we mean that the Mur D enzyme is not activated by salt species normally associated with the substrate (here D-glutamic acid or more preferably UDP-N-acetylmuramyl-L-alanine) or other assay components. Monovalent cations such as ammonium ($NH4^+$) and potassium (K^+) are particular salt species that activate Mur D. We note that if different amounts of substrate are used in an assay e.g. for K_m determinations or mode of inhibition studies, the amount of the activating cation such as ammonium ions is not constant. It can therefore not be clearly identified if an activity increase is due to activation or due to an increase of substrate concentration. Contrary to the results reported by Walsh et al. we have found that the *Staphylococcus aureus* Mur D enzyme is activated by cations such as $NH4^+$ and K^+ (cf. Figure 2).

Our analysis shows that activator-dependent forms of the MurD enzyme have the following common amino acid residues i.e. G96, A112, A116, V126, L129, M133, G296, P298 and V422. The indicated positions are based on the *E. faecalis* Mur D sequence as set out in Figure 8 (and corresponding sequence alignments).

Therefore in a further aspect of the invention we provide the use of an activator-independent MurD enzyme which contains a MurD amino acid sequence wherein one or more of the amino acid residues at the positions given above is not as indicated for that particular amino acid. More conveniently, at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least 8, or all nine of the amino acid residues are not as indicated.

The activator–independent MurD may conveniently contain an amino acid sequence comprising one of more of the following residues i.e. K96, C112, G116, T126, M129, L133, N296, S298 and I422. More conveniently, at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least 8, or all nine of the amino acid residues are as indicated.

The activator-independent MurD enzyme conveniently comprises the following *E. faecalis* amino acid sequence

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TEVELAGQIA ECPIVGITGT NGKTTTTTMI GLLLNADRTA GEARLAGNIG
FPASTVAQEA TAKDDLVMEL SSFQLMGIET FHPQIAVITN IFEAHLDYHG
SRKEYVAAKW AIQKNMTVED TLILNWNQVE LQTLAKTTAA NVLPFSTKEA
VEGAYLLDGK LYFNEEYIMP ADELGIPGSH NIENALAAIC VAKLKNVSNV
QIRQTLKNFS GVPHRTQFVG EVQQRRFYND SKATNILATE MALSGFDNQK
LLLLAGGLDR GNSFDELVPA LLGLKAIVLF GETKEKLAEA AKKANIETIL
FAENVQTAVT IAFDYSEKDD TILLSPACAS WDQYPNFEVR GEAFMQAVQQ
LKESEM

or a Mur D amino acid sequence having at least 85% homology, such as 90 or 95% homology, therewith.

Alternatively the activator-independent Mur D amino acid sequence has at least 70% sequence identity with the above amino acid sequence.

Whilst we do not wish to be limited by theoretical considerations we believe that the activator-independent Mur D enzyme may have up to 16, such as up to 16, 14, 12, 10, 8, 6, 4, or 2, amino acids removed from the N-terminus and/or up to 12, such as up to 12, 10, 8, 6, 4, or 2, amino acids removed from the C-terminus of the enzyme.

Activator-independent Mur D sequences that do not correspond to published Mur D enzyme sequences are novel and represent a further aspect of the present invention.

Any convenient screening assay format may be used. By way of non-limiting example we disclose the following:

The enzyme substrates are conveniently UDP-MurNac-L-Ala, D-Glutamate and ATP.

The enzyme is conveniently pre-incubated with the test compound to allow inhibitors to bind to the enzyme. This may allow the detection of inhibitors with a slow binding mode to the enzyme or allow detection of specifically modifying inhibitors that may be out-competed by the substrates.

Any appropriate buffer can be used that has a pKa in the pH range where *E.faecalis* MurD is active (pH 7.0 – 10.0) Examples of convenient buffers include buffers that do not contain phosphate such as GOOD Buffer i.e. Tris or Hepes (Good, et al. (1966) Biochemistry, 5, 467-477). Modulation of enzyme activity may be detected using any convenient detection system, such as those which include a colour change eg. using malachite green. These include absorbance spectrophotometers, absorbance plate reader or any other instrument that

can determine the absorption of a solution between for example a wavelength range of 400 to 800 nm

Modulation of enzyme activity can be inhibition or activation of enzyme activity, conveniently enzyme inhibition.

Appropriate control reactions are conveniently performed to determine if a chemical compound interferes with the detection system and/or has an absorbance at the detection wavelength.

The test compound is any convenient compound that may be useful in pharmaceutical research. It may be a polypeptide of equal to or greater than, 2 amino acids such as up to 6 amino acids, up to 10 or 12 amino acids, up to 20 amino acids or greater than 20 amino acids such as up to 50 amino acids. For drug screening purposes, preferred compounds are chemical compounds of low molecular weight and potential therapeutic agents. They are, for example of molecular mass less than about 1000 Daltons, such as less than 800, 600 or 400 Daltons. If desired, the test compound may be a member of a chemical library. This may comprise any convenient number of individual members, for example, tens to hundreds to thousands to millions etc., of suitable compounds, for example, peptides, peptoids and other oligomeric compounds (cyclic or linear), and template-based smaller molecules, for example, benzodiazepines, hydantoins, biaryls, carbocyclic and polycyclic compounds (eg. naphthalenes, phenothiazines, acridines, steroids etc.), carbohydrate and amino acids derivatives, dihydropyridines, benzhydryls and heterocycles (eg. triazines, indoles, thiazolidines etc.). The numbers quoted and the types of compounds listed are illustrative, but not limiting. Preferred chemical libraries comprise chemical compounds of low molecular weight and potential therapeutic agents.

In a further aspect of the invention we provide a MurD enzyme modulator resulting from use of the assay method of the invention.

The activator-independent MurD enzyme may be produced using known recombinant techniques for cloning and expression (cf. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Convenient expression systems for the MurD enzyme include T7 promoter-driven transcription of the *murD* gene in a suitable host, more conveniently *E. coli*. Examples of convenient expression vectors include those with a T7 promoter and suitable cloning sites such as pET28b and pET30a (Novagen Inc. Madison WI USA). The *E. coli* host strains used for expression in such a system include those that contain the T7 RNA polymerase gene that

can be induced to initiate transcription of the *murD* gene, more specifically BL21(DE3) and HMS174(DE3).

The invention will now be illustrated by reference to the following Specific Description and Figures wherein:

Figure 1 shows the lack of salt activation for *E. faecalis* MurD and shows that all salts but SO_4-ions (=inhibitor) have no effect on *E .faecalis* MurD

Figure 2 shows the salt activation of *S. aureus* MurD

Figure 3 shows that the *E. coli* enzyme activity is dependent on the presence of ammonium formate while the *E. faecalis* enzyme activity is independent on the presence of this salt.

Figure 4 shows the stability of *E. faecalis* Mur D in DMSO at concentrations of up to 5%.

Figure 5 shows a graph (Eyring Plot) with the activation enthalpies for several MurD orthologues, including *E. faecalis*. The higher this value the more temperature dependent the reaction rate

Figure 6 (a) and (b) show the pH dependence of several MurD orthologues, including *E. faecalis*

Figure 7 shows the background ATPase activity of several MurD orthologues, including *E. faecalis*.

Specific Description:

We have established that the *E. faecalis* MurD enzyme has the following additional advantages for screening purposes compared to MurD enzymes from other species.

(a) it is not affected up to a dimethylsulfoxide (DMSO) concentration of 5%. Since DMSO is commonly used in screening and IC_{50} measurements, this contributes to the stability of the assay results.

(b) its dependence of the reaction rate on temperature is low between 4°C to 40°C. Temperature variations during assays have only a minor effect on the enzyme activity.

(c) it has the broadest pH optimum (pH 7.5 – 9.5). Changes in pH due to compound addition is less likely to affect activity and therefore assay results

(d) it has a low background ATPase activity. An idle ATPase activity can create a background signal in a screen (phosphate is being produced whithout catalysis occuring) so a low amount of this activity is desirable to produce a sensitive assay.

We devised a screening assay for this ortholog using Malachite Green for detection of the phosphate product. The assay includes controls to check for compound interference with the signal and interference with the detection method.

1. Compounds are preincubated with *E.faecalis* MurD for 5-30 min.
2. The 3 substrates ATP, UDP-MurNac-L-Ala and D-Glutamate are added to initiate the reaction.
3. After 30-60 min the reaction is stopped with a Malachite Green solution. The signal is recorded spectrophotometrically 4-10 min after the Malachite Green soltion had been added.

To control for compound interference with the assay signal, compounds are submitted to the same proceedure but in the absence of *E. faecalis* MurD in step 1. Compounds interfering with the signal show an increased signal relative to a control where no *E. faecalis* MurD was present

To control for compound interference with the Malchite Green detection method, a constant amount of phosphate (10-15 uM) is replaced for *E. faecalis* MurD in step 1. Interference is detected by a increase or decrease of the signal relative to a control where no compound was present.

Specific assay conditions are 0.3nM *E. faecalis* MurD in 50mM Tris, 2.5mM DTT, 10mM $MgCl_2$, 0.01% Triton X-100, 50 uM ATP, 50uM UMA, 100uM D-Glu, pH 8.0. Typically

MurD is preincubated with inhibitor for 15 min in the absence of substrates. Subsequently the reaction is initiated by adding substrates and stopped by addition of Malachite Green after 60min. Signal is read 5 min after stopping the reaction. Although MurD activity is detected in this assay by formation of phosphate it is not limited to this detection method and can also be followed alternatively, for example, by measuring formation of other reaction products (UDP-N -acetylmuramyl-L-alanine-D-Glutamate, ADP) as well as the disappearance of the substrates (D-Glutamate, UDP-N-acetylmuramyl-L-alanine, ATP).

Claims:

1. The use of an activator-independent MurD enzyme in a screening assay to identify inhibitors of the enzyme, which assay comprises contacting the enzyme with a test compound in the presence of enzyme substrates and appropriate buffers and detecting any modulation of enzyme activity by the test compound.

2. The use as claimed in claim 1 wherein the activator-independent MurD enzyme comprises an amino acid sequence wherein one or more of the amino acid residues G96, A112, A116, V126, L129, M133, G296, P298 and V422 is a variant amino acid residue.

3. The use as claimed in claim 2 and wherein the activator–independent MurD enzyme comprises an amino acid sequence having one or more of the amino acid residues K96, C112, G116, T126, M129, L133, N296, S298 and I422.

4. The use as claimed in claim 1 and wherein the activator-independent MurD enzyme is an E. faecalis MurD enzyme.

5. The use as claimed in claim 1 and wherein the activator-independent MurD enzyme comprises the amino acid sequence
MKKITTYQNK KVLVLGLAKS GVSAAKLLHE LGALVTVNDA KQFDQNPDAQ DLLTLGIRVV TGGHPIELLD EEFELIVKNP GIPYTNPLVA EALTRKIPII TEVELAGQIA ECPIVGITGT NGKTTTTTMI GLLLNADRTA GEARLAGNIG FPASTVAQEA TAKDDLVMEL SSFQLMGIET FHPQIAVITN IFEAHLDYHG SRKEYVAAKW AIQKNMTVED TLILNWNQVE LQTLAKTTAA NVLPFSTKEA VEGAYLLDGK LYFNEEYIMP ADELGIPGSH NIENALAAIC VAKLKNVSNV QIRQTLKNFS GVPHRTQFVG EVQQRRFYND SKATNILATE MALSGFDNQK LLLLAGGLDR GNSFDELVPA LLGLKAIVLF GETKEKLAEA AKKANIETIL FAENVQTAVT IAFDYSEKDD TILLSPACAS WDQYPNFEVR GEAFMQAVQQ LKESEM or a sequence having at least 85% sequence identity therewith.

6. The use as claimed in any preceeding claim and wherein the activator-independent MurD enzyme has up to 16 amino acids removed from the N-terminus, or up to 12 amino acids removed from the C-terminus, of the amino acid sequence.

7. The use as claimed in any preceeding claim and wherein the enzyme substrates are UDP-MurNac-L-Ala, D-Glutamate and ATP.

8. The use as claimed in any preceeding claim and wherein the enzyme is pre-incubated with the test compound before contacting it with the substrates.

Figure 1

The lack of salt activation for *E. faecalis* MurD

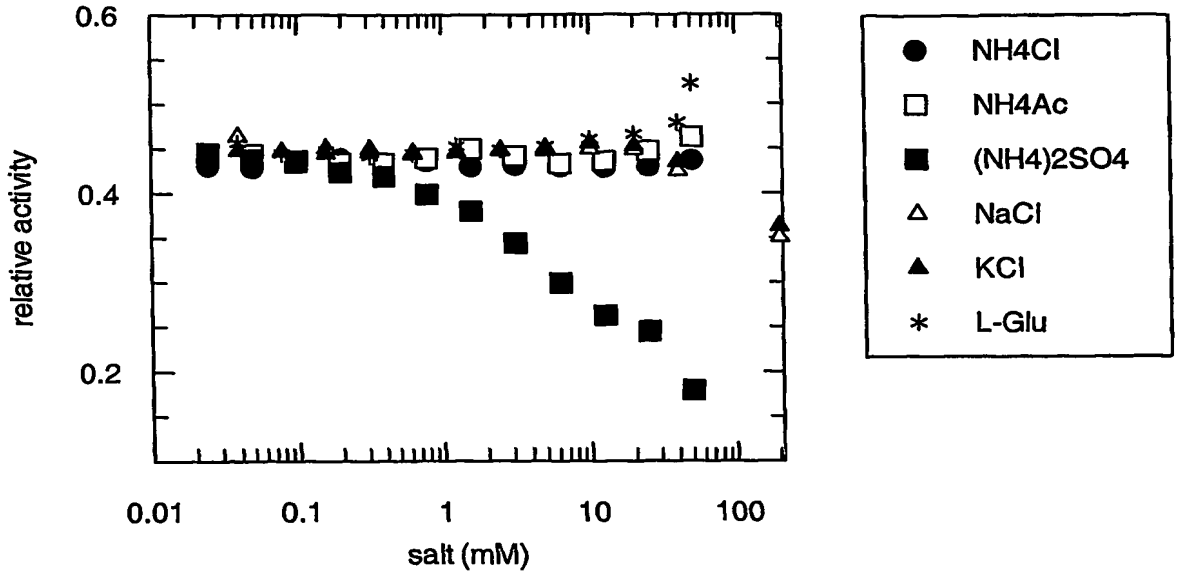
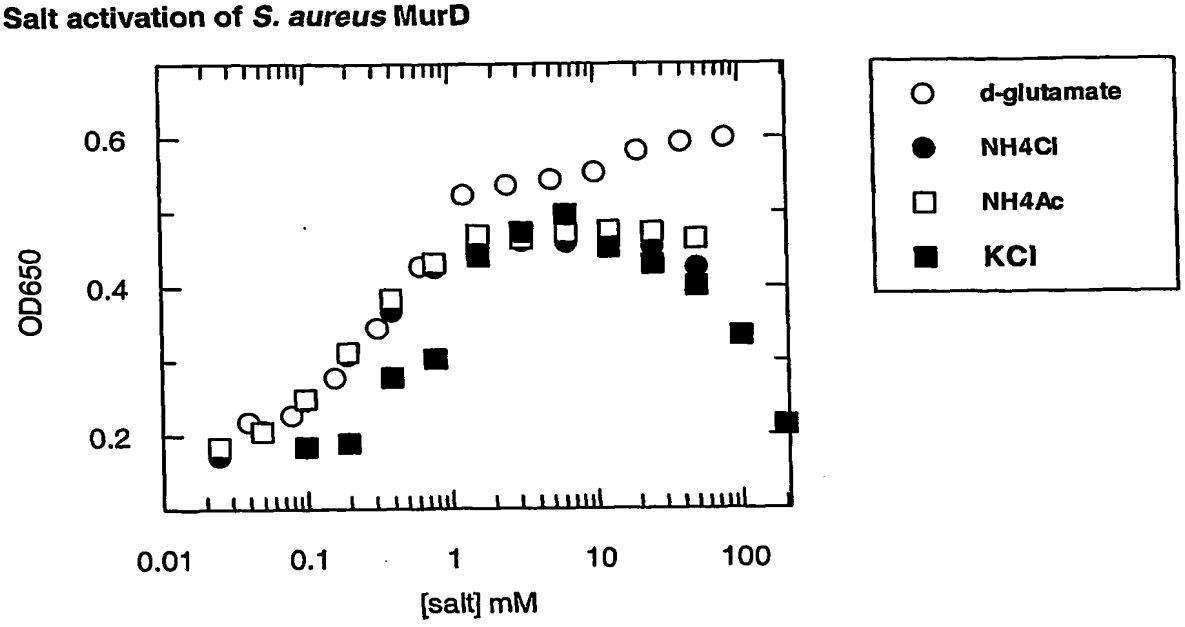


Figure 2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KCl

Figure 3

***E. coli* enzyme activity is dependent on ammonium formate**

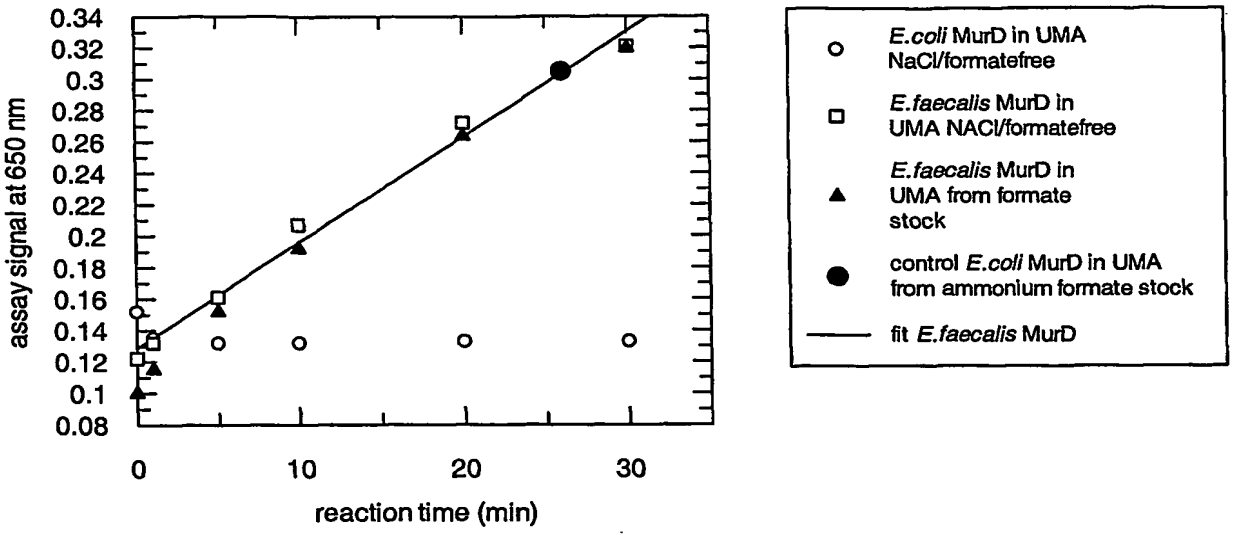


Figure 4

DMSO dependence of *E. faecalis* MurD

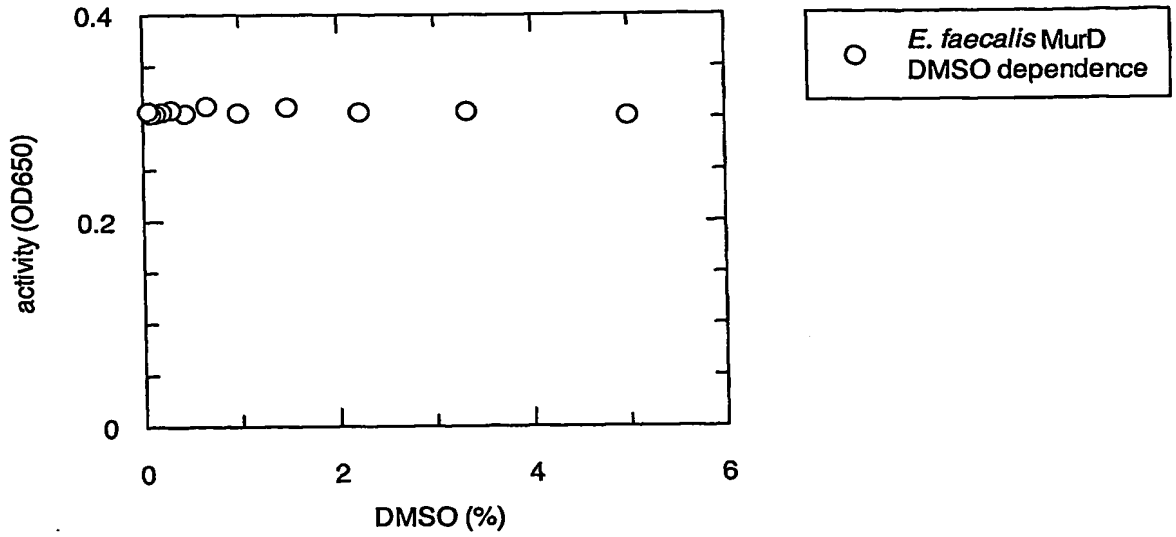


Figure 5

Temperature dependence of MurD orthologs

This Graph (Eyring Plot) shows the activation enthalpies for all for orthologs. The higher this value the more temperature dependent the reaction rate.

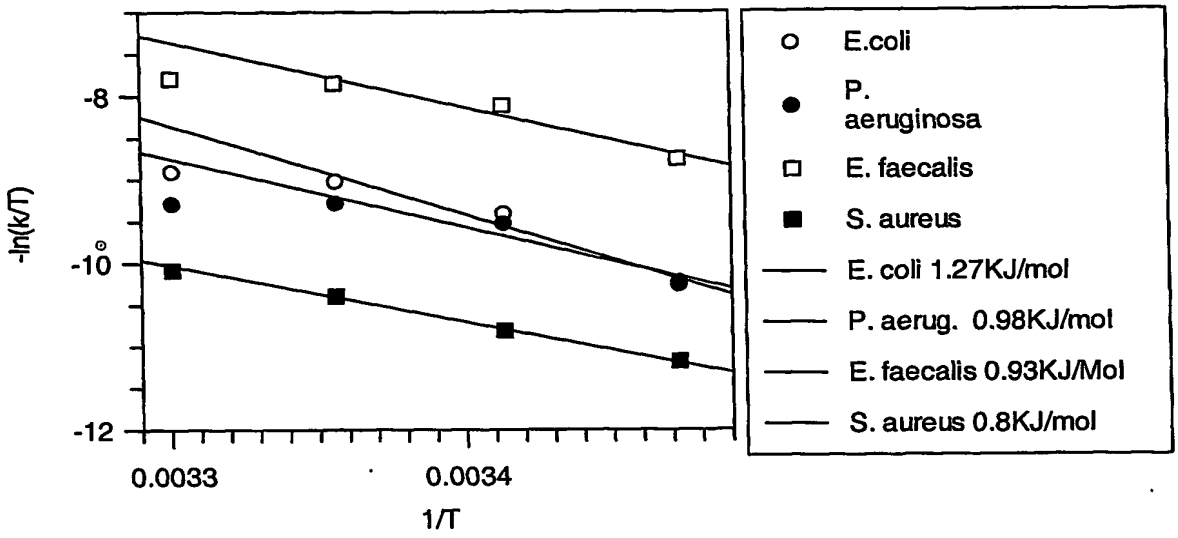
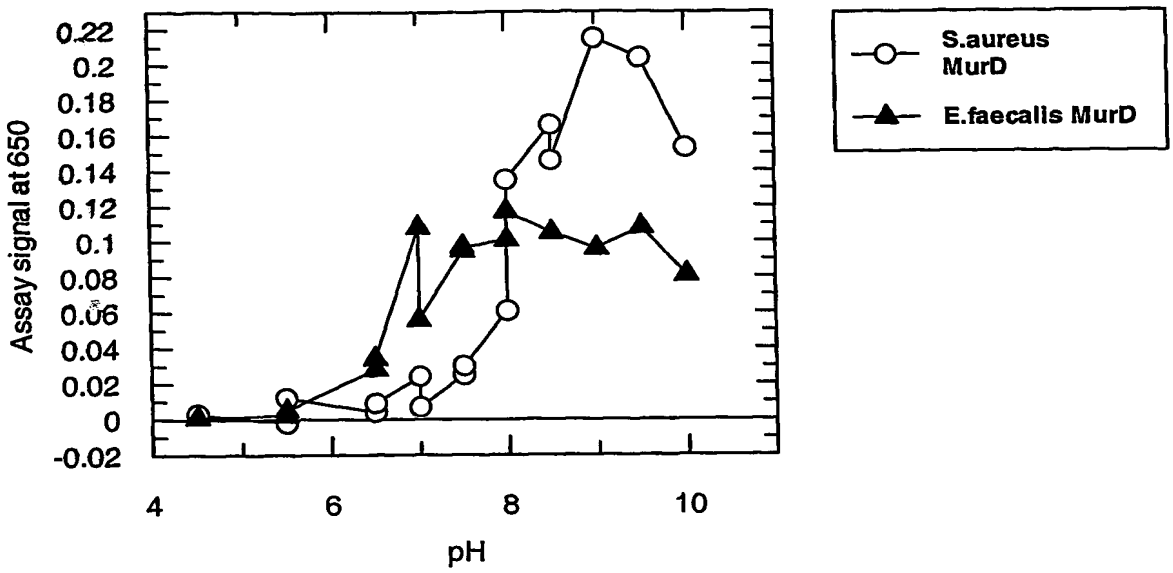


Figure 6

pH dependence of MurD orthologs

(a)



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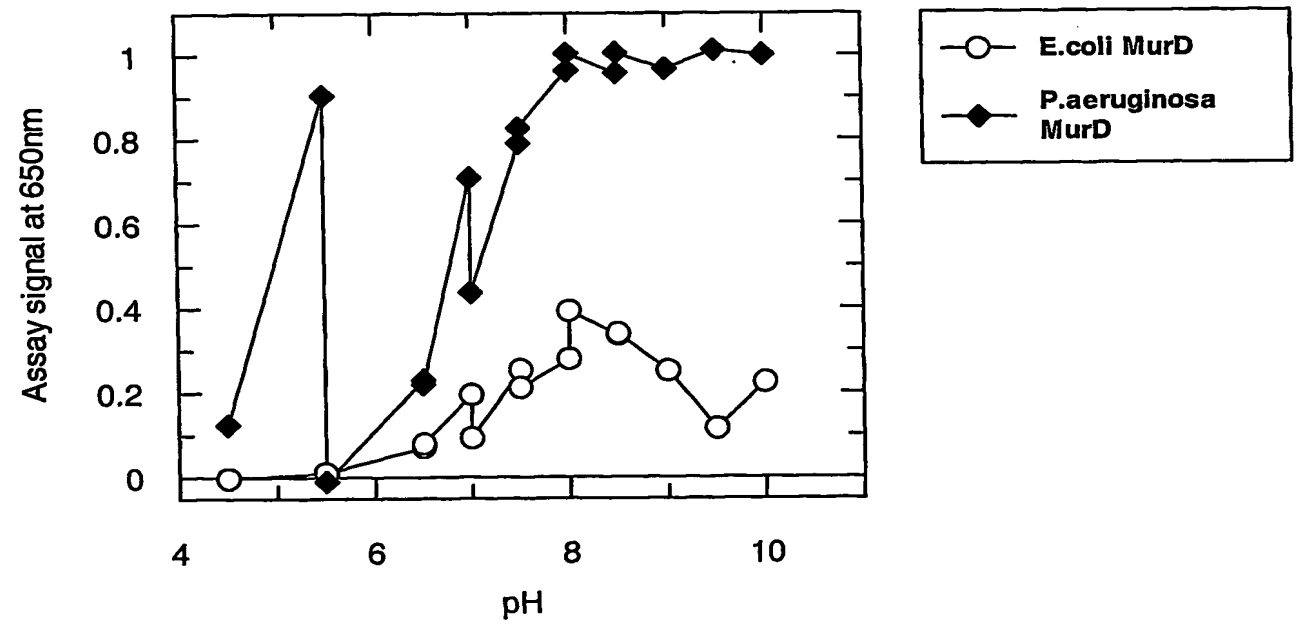
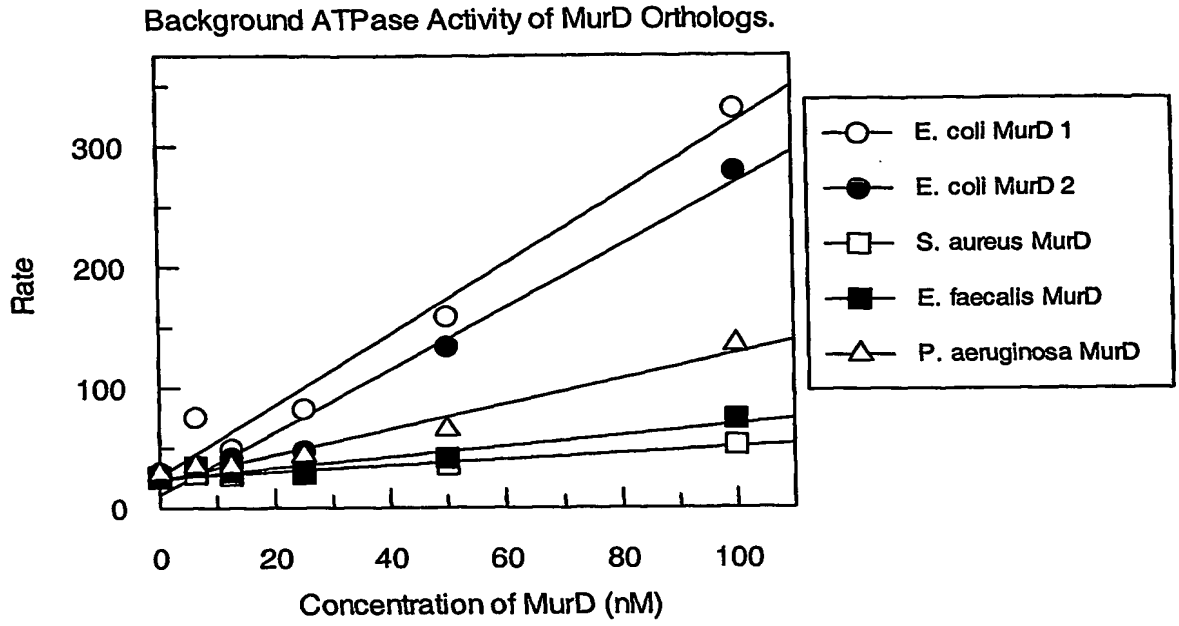


Figure 7

Background ATPase activity of MurD orthologs



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Gly Asn Ala Val Pro Leu Val Arg Val Ala Thr Leu Asp Glu Ala Val
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Leu Ile Gly Asp Met Phe Lys Lys Ser Arg Leu Thr Gly Xaa Xaa Arg
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Xaa Gln Pro Ile Ile Trp Leu Cys Gly Gly Leu Asp Xaa Arg Gly Asn
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Pro Ala Cys Ala Ser Trp Asp Gln Tyr Ser Thr Phe Glu Glu Arg Gly
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Glu Lys Phe Ile Glu Arg Phe Arg Ala His Leu Pro Ser Tyr Xaa
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5

INTERNATIONAL SEARCH REPORT

PCT/GB 03/04592

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/48 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALSH A W ET AL: "Comparison of the D-glutamate-adding enzymes from selected gram-positive and gram-negative bacteria" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 181, no. 17, September 1999 (1999-09), pages 5395-5401, XP002249293 ISSN: 0021-9193 section Materials and Methods; p.5397, right-hand column, paragraph (i) and (ii).	1-8
X	WO 99 23241 A (EL SHERBEINI MOHAMMED ;MERCK & CO INC (US); GEISSLER WAYNE M (US);) 14 May 1999 (1999-05-14) abstract page 16, line 9 - line 29	1,2

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2004	06/04/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Jacques, P

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INTERNATIONAL SEARCH REPORT

PCT/GB 03/04592

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 61050 A (MERCK & CO INC ;AZZOLINA BARBARA (US); EL SHERBEINI MOHAMED (US)) 2 December 1999 (1999-12-02) abstract page 17, line 14 - line 34	1,2
A	PUCCI MJ ET AL: "Identification and characterization of cell wall-cell division gene clusters in pathogenic gram-positive cocci" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 179, no. 17, September 1997 (1997-09), pages 5632-5635, XP002098344 ISSN: 0021-9193 the whole document	1-8

INTERNATIONAL SEARCH REPORT

PCT/GB 03/04592

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9923241 A	14-05-1999	CA 2308620 A1	14-05-1999
		EP 1034297 A1	13-09-2000
		JP 2001521750 T	13-11-2001
		WO 9923241 A1	14-05-1999
		US 6534284 B1	18-03-2003
WO 9961050 A	02-12-1999	CA 2333667 A1	02-12-1999
		EP 1079855 A1	07-03-2001
		EP 1082875 A1	14-03-2001
		JP 2002516076 T	04-06-2002
		JP 2002516780 T	11-06-2002
		WO 9961050 A1	02-12-1999